(54) **Assessing colorectal cancer**

(57) A method of assessing the presence or absence of colorectal cancer or the likely condition of a person believed to have colorectal cancer is conducted by analyzing the expression of a group of genes. Gene expression profiles in a variety of medium such as microarrays are included as are kits that contain them.

EP 1 355 149 A2

Description

BACKGROUND

[0001] This application claims the benefit of U.S Provisional Application No.60/368,798 filed on March 29, 2002.

[0002] This invention relates to diagnostics and prognostics for colorectal cancer based on the gene expression profiles of biological samples.

[0003] Colorectal cancer is a heterogenous disease, consisting of tumors thought to emerge through three major molecular mechanisms: 1) mutations in the adenomatous polyposis coli (APC) gene, or the β-catenin gene, combined with chromosomal instability, 2) mutations in DNA mismatch repair genes, such as MLH1, MSH2, PMS1, PMS2 and MSH6, associated with microsatellite instability and mutations in genes containing short repeats, and 3) gene silencing induced by hypermethylation of the promoter regions of tumor suppressor genes. The genetic complement of individual colorectal cancers is likely to include different combinations of genetic instability, specific mutations, and gene silencing. Chromosomal instability (CIN) is a common feature of cancers in general. It implies an aneuploid phenotype, in which whole chromosomes or large parts of them are being lost or gained. Microsomal instability (MIN) is found in diploid tumors with an increased mutation rate in short repeats. Both forms of genetic instability are common in colorectal cancer.

[0004] Colorectal cancers thus have complex origins and involve a number of interactions in different biological pathways. Serum markers, histological, and cytological examinations historically used to assist in providing diagnostic, prognostic, or therapy monitoring decisions often do not have desired reliability. Likewise, while use of a single genetic marker (e.g., increased expression of a particular gene) may be beneficial, the diversity of the cancers make it more likely that a portfolio of genetic markers is the best approach.

SUMMARY OF THE INVENTION

[0005] The invention is a method of assessing the presence or absence of colorectal cancer or the likely condition of a person believed to have colorectal cancer. In the method, a gene expression profile of a patient sample is analyzed to determine whether a patient has a colorectal cancer, whether a patient does not have colorectal cancer, whether a patient is likely to get colorectal cancer, or the response to treatment of a patient being treated for colorectal cancer.

[0006] Articles used in practising the methods are also an aspect of the invention. Such articles include gene expression profiles or representations of them that are fixed in machine-readable media such as computer readable media.

[0007] Articles used to identify gene expression profiles can also include substrates or surfaces, such as microarrays, to capture and/or indicate the presence, absence, or degree of gene expression.

DETAILED DESCRIPTION

[0008] The mere presence or absence of particular nucleic acid sequences in a tissue sample has only rarely been found to have diagnostic or prognostic value. Information about the expression of various proteins, peptides or mRNA, on the other hand, is increasingly viewed as important. The mere presence of nucleic acid sequences having the potential to express proteins, peptides, or mRNA (such sequences referred to as "genes") within the genome by itself is not determinative of whether a protein, peptide, or mRNA is expressed in a given cell. Whether or not a given gene capable of expressing proteins, peptides, or mRNA does so and to what extent such expression occurs, if at all, is determined by a variety of complex factors. Irrespective of difficulties in understanding and assessing these factors, assaying gene expression can provide useful information about the occurrence of important events such as tumero-genesis, metastasis, apoptosis, and other clinically relevant phenomena. Relative indications of the degree to which genes are active or inactive can be found in gene expression profiles. The gene expression profiles of this invention are used to diagnose and treat patients for colorectal cancer.

[0009] Sample preparation requires the collection of patient samples. Patient samples used in the inventive method are those that are suspected of containing diseased cells such as epithelial cells taken from a colon sample or from surgical margins. One useful technique for obtaining suspect samples is Laser Capture Microdisection (LCM). LCM technology provides a way to select the cells to be studied, minimizing variability caused by cell type heterogeneity. Consequently, moderate or small changes in gene expression between normal and cancerous cells can be readily detected. In a preferred method, the samples comprise circulating epithelial cells extracted from peripheral blood. These can be obtained according to a number of methods but the most preferred method is the magnetic separation technique described in U.S. Patent 6,136,182 assigned to Immunivest Corp which is incorporated herein by reference. Once the sample containing the cells of interest has been obtained, RNA is extracted and amplified and a gene expression profile is obtained, preferably via micro-array, for genes in the appropriate portfolios.

[0010] Preferred methods for establishing gene expression profiles include determining the amount of RNA that is produced by a gene that can code for a protein or peptide. This is accomplished by reverse transcriptase PCR (RT-PCR), competitive RT-PCR, real time RT-PCR, differential display RT-PCR, Northern Blot analysis and other related tests. While it is possible to conduct these techniques using individual PCR reactions, it is best to amplify complimentary DNA (cDNA) or complimentary RNA (cRNA) produced from mRNA and analyze it via microarray. A number of different array configurations and methods for their production are known to those of skill in the art and are described in U.S. Patents such as: 5,445,934; 5,532,128; 5,556,752; 5,242,974; 5,384,261; 5,405,783; 5,412,087; 5,424,186; 5,429,807; 5,436,327; 5,472,672; 5,527,681; 5,529,756; 5,545,531; 5,554,501; 5,561,071; 5,571,639; 5,593,839; 5,599,695; 5,624,711; 5,658,734; and 5,700,637; the disclosures of which are incorporated herein by reference.

[0011] Microarray technology allows for the measurement of the steady-state mRNA level of thousands of genes simultaneously thereby presenting a powerful tool for identifying effects such as the onset, arrest, or modulation of uncontrolled cell proliferation. Two microarray technologies are currently in wide use. The first are cDNA arrays and the second are oligonucleotide arrays. Although differences exist in the construction of these chips, essentially all downstream data analysis and output are the same. The product of these analyses are typically measurements of the intensity of the signal received from a labeled probe used to detect a cDNA sequence from the sample that hybridizes to a nucleic acid sequence at a known location on the microarray. Typically, the intensity of the signal is proportional to the quantity of cDNA, and thus mRNA, expressed in the sample cells. A large number of such techniques are available and useful. Preferred methods for determining gene expression can be found in US Patents 6,271,002 to Linsley, et al.; 6,218,122 to Friend, et al.; 6,218,114 to Peck, et al.; and 6,004,755 to Wang, et al., the disclosure of each of which is incorporated herein by reference.

[0012] Analysis of the expression levels is conducted by comparing such intensities. This is best done by generating a ratio matrix of the expression intensities of genes in a test sample versus those in a control sample. For instance, the gene expression intensities from a diseased tissue can be compared with the expression intensities generated from normal tissue of the same type (e.g., diseased colon tissue sample vs. normal colon tissue sample). A ratio of these expression intensities indicates the fold-change in gene expression between the test and control samples.

[0013] Gene expression profiles can also be displayed in a number of ways. The most common method is to arrange a raw fluorescence intensities or ratio matrix into a graphical dendogram where columns indicate test samples and rows indicate genes. The data is arranged so genes that have similar expression profiles are proximal to each other. The expression ratio for each gene is visualized as a color. For example, a ratio less than one (indicating down-regulation) may appear in the blue portion of the spectrum while a ratio greater than one (indicating up-regulation) may appear as a color in the red portion of the spectrum. Commercially available computer software programs are available to display such data including "GENESPRING" from Silicon Genetics, Inc. and "DISCOVERY" and "INFER" software from Partek, Inc.

[0014] Modulated genes used in the methods of the invention are shown in Table 1. The genes that are differentially expressed are shown as being either up regulated or down regulated in diseased cells. Up regulation and down regulation are relative terms meaning that a detectable difference (beyond the contribution of noise in the system used to measure it) is found in the amount of expression of the genes relative to some baseline. In this case, the baseline is the measured gene expression of a normal cell. The genes of interest in the diseased cells are then either up regulated or down regulated relative to the baseline level using the same measurement method. Diseased, in this context, refers to an alteration of the state of a body that interrupts or disturbs, or has the potential to disturb, proper performance of bodily functions as occurs with the uncontrolled proliferation of cells. Someone is diagnosed with a disease when some aspect of that person's genotype or phenotype is consistent with the presence of the disease. However, the act of conducting a diagnosis or prognosis includes the determination disease/status issues such as therapy monitoring. In therapy monitoring, clinical judgments are made regarding the effect of a given course of therapy by comparing the expression of genes over time to determine whether the gene expression profiles have changed or are changing to patterns more consistent with normal tissue.

[0015] Preferably, levels of up and down regulation are distinguished based on fold changes of the intensity measurements of hybridized microarray probes. A 2.0 fold difference is preferred for making such distinctions or a p-value less than .05. That is, before a gene is said to be differentially expressed in diseased versus normal cells, the diseased cell is found to yield at least 2 more, or 2 times less intensity than the normal cells. The greater the fold difference, the more preferred is use of the gene as a diagnostic. Genes selected for the gene expression profiles of the instant invention have expression levels that result in the generation of a signal that is distinguishable from those of the normal or non-modulated genes by an amount that exceeds background using clinical laboratory instrumentation.

[0016] Statistical values can be used to confidently distinguish modulated from non-modulated genes and noise. Statistical tests find the genes most significantly different between diverse groups of samples. The Student's t-test is an example of a robust statistical test that can be used to find significant differences between two groups. The lower the p-value, the more compelling the evidence that the gene is showing a difference between the different groups. Nevertheless, since microarrays measure more than one gene at a time, tens of thousands of statistical tests may be

asked at one time. Because of this, there is likelihood to see small p-values just by chance and adjustments for this using a Sidak correction as well as a randomization/permutation experiment can be made. A p-value less than .05 by the t-test is evidence that the gene is significantly different. More compelling evidence is a p-value less then .05 after the Sidak correct is factored in. For a large number of samples in each group, a p-value less than 0.05 after the randomization/permutation test is the most compelling evidence of a significant difference.

[0017] Another parameter that can be used to select genes that generate a signal that is greater than that of the non-modulated gene or noise is the use of a measurement of absolute signal difference. Preferably, the signal generated by the modulated gene expression is at least 20% different than those of the normal or non-modulated gene (on an absolute basis). It is even more preferred that such genes produce expression patterns that are at least 30% different than those of normal or non-modulated genes.

[0018] Genes can be grouped so that information obtained about the set of genes in the group provides a sound basis for making a clinically relevant judgment such as a diagnosis, prognosis, or treatment choice. These sets of genes make up the portfolios of the invention. In this case, the judgments supported by the portfolios involve colorectal cancer. Portfolios of gene expression profiles can be comprised of combinations of genes described in Example 3. As with most diagnostic markers, it is often desirable to use the fewest number of markers sufficient to make a correct medical judgment. This prevents a delay in treatment pending further analysis as well inappropriate use of time and resources. In this case, such a minimal portfolio can be comprised of a combination of genes from Example 4.

[0019] Preferably, portfolios are established such that the combination of genes in the portfolio exhibit improved sensitivity and specificity relative to individual genes or randomly selected combinations of genes. In the context of the instant invention, the sensitivity of the portfolio can be reflected in the fold differences exhibited by a gene's expression in the diseased state relative to the normal state. Specificity can be reflected in statistical measurements of the correlation of the signaling of gene expression with the condition of interest. For example, standard deviation can be a used as such a measurement. In considering a group of genes for inclusion in a portfolio, a small standard deviation in expression measurements correlates with greater specificity. Other measurements of variation such as correlation coefficients can also be used in this capacity. The most preferred method of establishing gene expression portfolios is through the use of optimization algorithms such as the mean variance algorithm widely used in establishing stock portfolios. This method is described in detail in the co-pending patent application entitled "Portfolio Selection" by Tim Jatkoe, et. al., of equal date hereto. Essentially, the method calls for the establishment of a set of inputs (stocks in financial applications, expression as measured by intensity here) that will optimize the return (e.g., signal that is generated) one receives for using it while minimizing the variability of the return. Many commercial software programs are available to conduct such operations. "Wagner Associates Mean-Variance Optimization Application", referred to as "Wagner Software" throughout this specification, is preferred. This software uses functions from the "Wagner Associates Mean-Variance Optimization Library" to determine an efficient frontier and optimal portfolios in the Markowitz sense is preferred.

[0020] Use of this type of software requires that microarray data be transformed so that it can be treated as an input in the way stock return and risk measurements are used when the software is used for its intended financial analysis purposes. For example, when Wagner Software is employed in conjunction with microarray intensity measurements the following data transformation method is employed.

[0021] Genes are first pre-selected by identifying those genes whose expression shows at least some minimal level of differentiation. The preferred pre-selection process is conducted as follows. A baseline class is selected. Typically, this will comprise genes from a population that does not have the condition of interest. For example, if one were interested in selecting a portfolio of genes that are diagnostic for breast cancer, samples from patients without breast cancer can be used to make the baseline class. Once the baseline class is selected, the arithmetic mean and standard deviation is calculated for the indicator of gene expression of each gene for baseline class samples. This indicator is typically the fluorescent intensity of a microarray reading. The statistical data computed is then used to calculate a baseline value of (X*Standard Deviation + Mean) for each gene. This is the baseline reading for the gene from which all other samples will be compared. X is a stringency variable selected by the person formulating the portfolio. Higher values of X are more stringent than lower. Preferably, X is in the range of .5 to 3 with 2 to 3 being more preferred and 3 being most preferred.

[0022] Ratios between each experimental sample (those displaying the condition of interest) versus baseline readings are then calculated. The ratios are then transformed to base 10 logarithmic values for ease of data handling by the software. This enables down regulated genes to display negative values necessary for optimization according to the Markman mean-variance algorithm using the Wagner Software.

[0023] The preprocessed data comprising these transformed ratios are used as inputs in place of the asset return values that are normally used in the Wagner Software when it is used for financial analysis purposes.

[0024] Once an efficient frontier is formulated, an optimized portfolio is selected for a given input level (return) or variance that corresponds to a point on the frontier. These inputs or variances are the predetermined standards set by the person formulating the portfolio. Stated differently, one seeking the optimum portfolio determines an acceptable

input level (indicative of sensitivity) or a given level of variance (indicative of specificity) and selects the genes that lie along the efficient frontier that correspond to that input level or variance. The Wagner Software can select such genes when an input level or variance is selected. It can also assign a weight to each gene in the portfolio as it would for a stock in a stock portfolio.

[0025] Determining whether a sample has the condition for which the portfolio is diagnostic can be conducted by comparing the expression of the genes in the portfolio for the patient sample with calculated values of differentially expressed genes used to establish the portfolio. Preferably, a portfolio value is first generated by summing the multiples of the intensity value of each gene in the portfolio by the weight assigned to that gene in the portfolio selection process. A boundary value is then calculated by (Y*standard deviation + mean of the portfolio value for baseline groups) where Y is a stringency value having the same meaning as X described above. A sample having a portfolio value greater than the portfolio value of the baseline class is then classified as having the condition. If desired, this process can be conducted iteratively in accordance with well known statistical methods for improving confidence levels.

[0026] Optionally one can reiterate this process until best prediction accuracy is obtained.

[0027] The process of portfolio selection and characterization of an unknown is summarized as follows:

1. Choose baseline class.
2. Calculate mean, and standard deviation of each gene for baseline class samples.
3. Calculate (X*Standard Deviation + Mean) for each gene. This is the baseline reading from which all other samples will be compared. X is a stringency variable with higher values of X being more stringent than lower.
4. Calculate ratio between each Experimental sample versus baseline reading calculated in step 3.
5. Transform ratios such that ratios less than 1 are negative (eg.using Log base 10). (Down regulated genes now correctly have negative values necessary for MV optimization).
6. These transformed ratios are used as inputs in place of the asset returns that are normally used in the software application.
7. The software will plot the efficient frontier and return an optimized portfolio at any point along the efficient frontier.
8. Choose a desired return or variance on the efficient frontier.
9. Calculate the Portfolio's Value for each sample by summing the multiples of each gene's intensity value by the weight generated by the portfolio selection algorithm.
10. Calculate a boundary value by adding the mean Portfolio Value for Baseline groups to the multiple of Y and the Standard Deviation of the Baseline's Portfolio Values. Values greater than this boundary value shall be classified as the Experimental Class.
11. Optionally one can reiterate this process until best prediction accuracy is obtained.

[0028] Alternatively, genes can first be pre-selected by identifying those genes whose expression shows some minimal level of differentiation. The pre-selection in this alternative method is preferably based on a threshold given by

$$1 \leq \left| \frac{(\mu_t - \mu_n)}{(\sigma_t + \sigma_n)} \right|,$$

where μ_t is the mean of the subset known to possess the disease or condition, μ_n is the mean of the subset of normal samples, and $\sigma_t + \sigma_n$ represent the combined standard deviations. A signal to noise cutoff can also be used by pre-selecting the data according to a relationship such as

$$0.5 \leq \left| \frac{(\mu_t - MAX_n)}{(\sigma_t + \sigma_n)} \right|.$$

This ensures that genes that are pre-selected based on their differential modulation are differentiated in a clinically significant way. That is, above the noise level of instrumentation appropriate to the task of measuring the diagnostic parameters. For each marker pre-selected according to these criteria, a matrix is established in which columns represents samples, rows represent markers and each element is a normalized intensity measurement for the expression of that marker according to the relationship:

$$\left| \frac{(\mu_t - I)}{\mu_t} \right|$$

where I is the intensity measurement.

[0029] It is also possible to set additional boundary conditions to define the optimal portfolios. For example, portfolio size can be limited to a fixed range or number of markers. This can be done either by making data pre-selection criteria more stringent (e.g,

$$.8 \leq \left| \frac{(\mu_t - MAX_n)}{(\sigma_t + \sigma_n)} \right|$$

instead of

$$0.5 \leq \left| \frac{(\mu_t - MAX_n)}{(\sigma_t + \sigma_n)} \right|$$

or by using programming features such as restricting portfolio size. One could, for example, set the boundary condition that the efficient frontier is to be selected from among only the most optimal 10 genes. One could also use all of the genes pre-selected for determining the efficient frontier and then limit the number of genes selected (e.g., no more than 10).

[0030] The process of selecting a portfolio can also include the application of heuristic rules. Preferably, such rules are formulated based on biology and an understanding of the technology used to produce clinical results. More preferably, they are applied to output from the optimization method. For example, the mean variance method of portfolio selection can be applied to microarray data for a number of genes differentially expressed in subjects with breast cancer. Output from the method would be an optimized set of genes that could include some genes that are expressed in peripheral blood as well as in diseased breast tissue. If sample used in the testing method are obtained from peripheral blood and certain genes differentially expressed in instances of breast cancer could also be differentially expressed in peripheral blood, then a heuristic rule can be applied in which a portfolio is selected from the efficient frontier excluding those that are differentially expressed in peripheral blood. Of course, the rule can be applied prior to the formation of the efficient frontier by, for example, applying the rule during data pre-selection.

[0031] Other heuristic rules can be applied that are not necessarily related to the biology in question. For example, one can apply the rule that only a given percentage of the portfolio can be represented by a particular gene or genes. Commercially available software such as the Wagner Software readily accommodates these types of heuristics. This can be useful, for example, when factors other than accuracy and precision (e.g., anticipated licensing fees) have an impact on the desirability of including one or more genes.

[0032] One method of the invention involves comparing gene expression profiles for various genes (or portfolios) to conduct diagnoses as described above. The gene expression profiles of each of the genes comprising the portfolio are fixed in a medium such as a computer readable medium. This can take a number of forms. For example, a table can be established into which the range of signals (e.g., intensity measurements) indicative of disease is input. Actual patient data can then be compared to the values in the table to determine whether the patient samples are normal or diseased. In a more sophisticated embodiment, patterns of the expression signals (e.g., flourescent intensity) are recorded digitally or graphically. The gene expression patterns from the gene portfolios used in conjunction with patient samples are then compared to the expression patterns. Pattern comparison software can then be used to determine whether the patient samples have a pattern indicative of the disease in question. Of course, these comparisons can also be used to determine whether the patient results are normal. The expression profiles of the samples are then compared to the portfolio of a normal or control cell. If the sample expression patterns are consistent with the expression pattern for a colorectal cancer then (in the absence of countervailing medical considerations) the patient is diagnosed as positive for colorectal cancer. If the sample expression patterns are consistent with the expression pattern from the normal/control cell then the patient is diagnosed negative for colorectal cancer.

[0033] Numerous well known methods of pattern recognition are available. The following references provide some examples:

Weighted Voting: Golub, TR., Slonim, DK., Tamaya, P., Huard, C., Gaasenbeek, M., Mesirov, JP., Coller, H., Loh, L., Downing, JR., Caligiuri, MA., Bloomfield, CD., Lander, ES. *Molecular classification of cancer: class discovery and class prediction by gene expression monitoring.* Science 286:531-537, 1999

Support Vector Machines: Su, AI., Welsh, JB., Sapinoso, LM., Kern, SG., Dimitrov, P., Lapp, H., Schultz, PG., Powell, SM., Moskaluk, CA., Frierson, HF. Jr., Hampton, GM. *Molecular classification of human carcinomas by use of gene expression signatures.* Cancer Research 61:7388-93, 2001 and

Ramaswamy, S., Tamayo, P., Rifkin, R., Mukherjee, S., Yeang, CH., Angelo, M., Ladd, C., Reich, M., Latu-

lippe, E., Mesirov, JP., Poggio, T., Gerald, W., Loda, M., Lander, ES., Gould, TR. *Multiclass cancer diagnosis using tumor gene expression signatures* Proceedings of the National Academy of Sciences of the USA 98:15149-15154, 2001

K-nearest Neighbors: Ramaswamy, S., Tamayo, P., Rifkin, R., Mukherjee, S., Yeang, CH., Angelo, M., Ladd, C., Reich, M., Latulippe, E., Mesirov, JP., Poggio, T., Gerald, W., Loda, M., Lander, ES., Gould, TR. *Multiclass cancer diagnosis using tumor gene expression signatures* Proceedings of the National Academy of Sciences of the USA 98:15149-15154, 2001

Correlation Coefficients: van 't Veer LJ, Dai H, van de Vijver MJ, He YD, Hart AA, Mao M, Peterse HL, van der Kooy K, Marton MJ, Witteveen AT, Schreiber GJ, Kerkhoven RM, Roberts C, Linsley PS, Bernards R, Friend SH. Gene expression profiling predicts clinical outcome of breast cancer. Nature. 2002 Jan 31;415(6871):530-6.

[0034]   The gene expression profiles of this invention can also be used in conjunction with other non-genetic diagnostic methods useful in cancer diagnosis, prognosis, or treatment monitoring. For example, in some circumstances it is beneficial to combine the diagnostic power of the gene expression based methods described above with data from conventional markers such as serum protein markers (e.g., carcinoembryonic antigen). A range of such markers exists including such analytes as CA19-9, CA 125, CK-BB, and Guanylyl Cyclase C. In one such method, blood is periodically taken from a treated patient and then subjected to an enzyme immunoassay for one of the serum markers described above. When the concentration of the marker suggests the return of tumors or failure of therapy, a sample source amenable to gene expression analysis is taken. Where a suspicious mass exists, a fine needle aspirate is taken and gene expression profiles of cells taken from the mass are then analyzed as described above. Alternatively, tissue samples may be taken from areas adjacent to the tissue from which a tumor was previously removed. This approach can be particularly useful when other testing produces ambiguous.

[0035]   Combining the use of genetic markers with other diagnostics is most preferred when the reliability of the other diagnostic is suspect. For example, it is known that serum levels of CEA can be substantially affected by factors having nothing to do with a patient's cancer status. It can be beneficial to conduct a combination gene expression/CEA assay when a patient being monitored following treatment for colon cancer shows heightened levels of routine CEA assays.

[0036]   Articles of this invention include representations of the gene expression profiles useful for treating, diagnosing, prognosticating, and otherwise assessing diseases. These profile representations are reduced to a medium that can be automatically read by a machine such as computer readable media (magnetic, optical, and the like). The articles can also include instructions for assessing the gene expression profiles in such media. For example, the articles may comprise a CD ROM having computer instructions for comparing gene expression profiles of the portfolios of genes described above. The articles may also have gene expression profiles digitally recorded therein so that they may be compared with gene expression data from patient samples. Alternatively, the profiles can be recorded in different representational format. A graphical recordation is one such format. Clustering algorithms such as those incorporated in "GENESPRING" and "DISCOVER" computer programs mentioned above can best assist in the visualization of such data.

[0037]   Different types of articles of manufacture according to the invention are media or formatted assays used to reveal gene expression profiles. These can comprise, for example, microarrays in which sequence complements or probes are affixed to a matrix to which the sequences indicative of the genes of interest combine creating a readable determinant of their presence. Alternatively, articles according to the invention can be fashioned into reagent kits for conducting hybridization, amplification, and signal generation indicative of the level of expression of the genes of interest for detecting colorectal cancer.

[0038]   Kits made according to the invention include formatted assays for determining the gene expression profiles. These can include all or some of the materials needed to conduct the assays such as reagents and instructions.

[0039]   The invention is further illustrated by the following non-limiting examples.

Examples: Genes analyzed according to this invention are identified by reference to Gene ID Numbers in the GenBank database. These are typically related to full-length nucleic acid sequences that code for the production of a protein or peptide. One skilled in the art will recognize that identification of full-length sequences is not necessary from an analytical point of view. That is, portions of the sequences or ESTs can be selected according to well-known principles for which probes can be designed to assess gene expression for the corresponding gene.

Example 1- Sample Handling and LCM.

[0040]   Twenty-seven fresh frozen tissue samples were collected from patients who had surgery for a colorectal tumor. Nineteen of the samples were colorectal malignancy specimens, and eight of the samples were of normal colon mucosa. The tissues were snap frozen in liquid nitrogen within 20-30 minutes of harvesting, and stored at -80C° thereafter. For laser capture, the samples were cut (6μm), and one section was mounted on a glass slide, and the second on film (P. A.L.M.), which had been fixed onto a glass slide (Micro Slides Colorfrost, VWR Scientific, Media, PA). The section

mounted on a glass slide was after fixed in cold acetone, and stained with Mayer's Haematoxylin (Sigma, St. Louis, MO). A pathologist analyzed the samples for diagnosis and grade. The clinical stage was estimated from the accompanying surgical pathology and clinical reports, using the Dukes classification. The section mounted on film was after fixed for five minutes in 100% ethanol, counter stained for 1 minute in eosin/100% ethanol (100μg of Eosin in 100ml of dehydrated ethanol), quickly soaked once in 100% ethanol to remove the free stain, and air dried for 10 minutes.

[0041] Two of the colorectal adenocarcinomas were of grade 1, 10 of grade 2, and 5 of grade 3. One of the malignant samples was a carcinoid tumor of the caecum, and one a metastatic melanoma lesion. Two of the adenocarcinoma samples represented the mucinous subtype, and one the signet cell subtype. The Dukes staging of the adenocarcinomas divided them as follows: Dukes A: 2, Dukes B: 5, Dukes C: 7, Dukes D: 3. Six of the adenocarcinomas had been irradiated preoperatively.

[0042] Before use in LCM, the membrane (LPC-MEMBRANE PEN FOIL 1.35 μm No 8100, P.A.L.M. GmbH Mikrolaser Technologie, Bernried, Germany) and slides were pretreated to abolish RNases, and to enhance the attachment of the tissue sample onto the film. Briefly, the slides were washed in DEP H_2O, and the film was washed in RNase AWAY (Molecular Bioproducts, Inc., San Diego, CA) and rinsed in DEP H_2O. After attaching the film onto the glass slides, the slides were baked at +120°C for 8 hours, treated with TI-SAD (Diagnostic Products Corporation, Los Angeles, CA, 1:50 in DEP H_2O, filtered through cotton wool), and incubated at +37°C for 30 minutes. Immediately before use, a 10μl aliquot of RNase inhibitor solution (Rnasin Inhibitor 2500U=33U/μl N211A, Promega GmbH, Mannheim, Germany, 0.5μl in 400μl of freezing solution, containing 0.15 mol NaCl, 10 mmol Tris pH 8.0, 0.25 mmol dithiothreitol) was spread onto the film, where the tissue sample was to be mounted.

[0043] The tissue sections mounted on film were used for LCM. Approximately 2000 epithelial cells/sample were captured using the PALM Robot-Microbeam technology (P.A.L.M. Mikrolaser Technologie, Carl Zeiss, Inc., Thornwood, NY), coupled into Zeiss Axiovert 135 microscope (Carl Zeiss Jena GmbH, Jena, Germany). The surrounding stroma in the normal mucosa, and the occasional intervening stromal components in cancer samples, were included. The captured cells were put in tubes in 100% ethanol and preserved at -80°C.

Example 2- RNA Extraction and Amplification.

[0044] Zymo-Spin Column (Zymo Research, Orange, CA 92867) was used to extract total RNA from the LCM captured samples. About 2 ng of total RNA was resuspended in 10 ul of water and 2 rounds of the T7 RNA polymerase based amplification were performed to yield about 50 ug of amplified RNA.

Example 3- cDNA Microarray Hybridization and Quantitation.

[0045] A set of cDNA microarrays consisting of approximately 20,000 human cDNA clones was used to test the samples. About 30 plant genes were also printed on the microarrays as a control for non-specific hybridization. Cy3-labeled cDNA probes were synthesized from 5 ug of aRNA of the LCM captured cells. The probes were purified with Qiagen's Nucleotide Removal Columns and then hybridized to the microarrays for 14-16 hours. The slides were washed and air-dried before scanning. cDNA microarrays were scanned for cy3 fluorescence and ImaGene software (Biodiscovery, Los Angeles, CA) was used for quantitation. For each cDNA clone, four measurements were obtained using duplicate spots and duplicate arrays and the intensities were averaged.

[0046] cDNAs were printed on amino silane-coated slides (Coming) with a Generation III Micro-array Spotter (Molecular Dynamics). The cDNAs were PCR amplified, purified (Qiagen PCR purification kit), and mixed 1:1 with 10 M NaSCN printing buffer. Prior to hybridization micro-arrays were incubated in isopropanol at room temperature for 10 min. The probes were incubated at 95°C for 2 min, at room temperature for 5 min, and then applied to three replicate slides. Cover slips were sealed onto the slides with DPX (Fluka) and incubated at 42°C overnight. Slides were then washed at 55°C for 5min in 1X SSC/0.2% SDS and 0.1X SSC/0.2% SDS, dipped in 0.1X SSC and dried before being scanned by a GenIII Array Scanner (Molecular Dynamics). The fluorescence intensity for each spot was analyzed with AUTOGENE software (Biodiscovery, Los Angeles).

[0047] Chip intensities were linearly normalized forcing the intensity reading at the 75th percentile equivalent to a value of 100 on each chip. Every gene on the chip was normalized to itself by dividing the intensity reading for that gene by the median of the gene's expression value readings over all the samples. Prior to clustering, genes that did not have an intensity reading of 100 or greater in at least one sample were filtered out in order to limit the background affect on the similarity metrics. A set of 6,225 genes was selected for clustering analysis. Hierarchical clustering was performed using correlation as) a measure of similarity, which groups together samples with genes that are showing positive changes at the same time without any consideration for negative changes (Silicon Genetics, Sunnyville, CA). Each of the major nodes in the dendrogram was then considered a subgroup of samples. Differentially expressed genes were identified by comparing each tumor subgroup to the normal group. The selection was based on a signal to noise measurement threshold given by

$$1 \le \left| \frac{(\mu_t - \mu_n)}{(\sigma_t + \sigma_n)} \right|,$$

where μ_t is the mean of the tumor subset, μ_n is the mean of the subset of normal samples, and $\sigma_t + \sigma_n$ represent the combined standard deviations. The within-group coefficient of variation of the intensity readings of a gene had to be less than 0.33, for the gene to be included in the pair-wise comparisons. The median of the tumor group over the median of the normal group had to) be greater than, or equal to 2 for up-regulation, and less than, or equal to 0.5 for down-regulation. If a gene met all the criteria, it was selected. The genes selected in all the comparisons were considered consistently dysregulated in colorectal cancer. The p-values for the statistical significance were calculated using a T-test assuming unequal variance. The gene set for clustering was also subjected to principal component analysis (PCA) using a software package (Partek, St Louis, MO). The data was then projected onto the reduced 3-dimensional space. The normal and tumor colorectal samples were represented by the projected expression levels.

[0048] A list of genes with large up-regulated differentials was created to distinguish between the tumor and normal samples. One-hundred and twenty-three genes were preselected by using

$$0.5 \le \left| \frac{(\mu_t - MAX_n)}{(\sigma_t + \sigma_n)} \right|$$

as a signal to noise cutoff. A ratio equal to, or greater than 1.5 was the minimal criterion for up-regulation. Genes were also included if

$$0.9 \le \left| \frac{(\mu_t - \mu_n)}{(\sigma_t + \sigma_n)} \right|.$$

A portfolio of four genes was established, each having at least a three fold expression differential between tumor and normal cells.

[0049] Differentially Expressed Genes in Colorectal Cancer. Thirty-nine genes were differentially expressed in all tumor samples as compared to normal colon mucosa. Thirty-seven of them were significantly down-regulated in all the tumors, except for an outlier. Two of them were up-regulated. The identities of the genes were verified by sequencing the cDNA clones placed on the microarray. Results are shown in Table 1.

Table 1

Modulated Genes					
ACCESSION	GENE DESCRIPTION	MEAN SIGNAL INTENSITY (NORMAL)	MEAN SIGNAL INTENSITY (TUMOR)	P-VALUE	
AF071569	CaM kinase II gene subtype delta 2.	93	39	4.64E-09	Seq. ID No. 1
AB014530	Homo sapiens mRNA for KIAA0630 protein	108	50	4.83E-07	Seq. ID No.2
AK000319	Human cDNA KIAA0630	236	69	7.84E-06	Seq. ID No.3
U81504	beta-3A-adaptin subunit of the AP-3 complex mRNA,	241	75	3.52E-05	Seq. ID No.4
AB011166	Human cDNA KIAA0594	116	55	3.53E-05	Seq. ID No.5
AB040914	Human cDNA KIAA1481	187	59	8.85E-05	Seq. ID No.6

Table 1 (continued)

Modulated Genes					
ACCESSION	GENE DESCRIPTION	MEAN SIGNAL INTENSITY (NORMAL)	MEAN SIGNAL INTENSITY (TUMOR)	P-VALUE	
AK025205	Human cDNA FLJ21552	322	97	0.00013	Seq. ID No.7
AJ278219	Fatty acid hydroxylase	143	53	0.00011	Seq. ID No.8
AB046854	Human cDNA KIAA1634	142	59	0.00020	Seq. ID No.9
R00585	Unknown	149	57	1.28E-09	Seq. ID No.10
S45844	Spi-B transcription factor	140	43	0.00043	Seq. ID No.11
X98311	Carcinoembryonic antigen family member 2 (CGM2)	6137	223	0.00044	Seq. ID No.12
BAA78050	NADPH oxidoreductase homolog	153	84	0.00048	Seq. ID No.40
N72128	Unknown	164	77	0.00068	Seq. ID No.13
AB040955	Human cDNA KIAA1552	334	120	0.00067	Seq. ID No.14
AF125101	HSPC040 protein	363	115	0.0011	Seq. ID No.15
AB023229	Human cDNA KIAA1012	263	88	0.00099	Seq. ID No.16
N95761	a-L-fucosidase gene	429	104	0.00047	Seq. ID No.17
AK025033	Human cDNA FLJ21380	180	85	0.0010	Seq. ID No.18
L10844	Human cellular growth regulating protein	206	101	0.0013	Seq. ID No.19
H96534	H.sapiens mRNA for gp25L2 protein.	147	58	0.0015	Seq. ID No.20
AK001521	Human cDNA FLJ10659	157	53	0.0019	Seq. ID No.21
AF151039	HSPC205 protein	117	60	0.0017	Seq. ID No.22
AF052059	SEL 1L protein	168	53	0.0016	Seq. ID No.23
N24597	Unknown	166	62	0.0016	Seq. ID No.24
AK001950	Inner centromere protein	148	64	0.0029	Seq. ID No.25
BAA02649	Macrophage scavenger receptor type I	118	44	0.0031	Seq. ID No.41
N75004	Unknown	98	48	0.0031	Seq. ID No.26

Table 1   (continued)

Modulated Genes					
ACCESSION	GENE DESCRIPTION	MEAN SIGNAL INTENSITY (NORMAL)	MEAN SIGNAL INTENSITY (TUMOR)	P-VALUE	
W16916	Human cDNA KIAA0260	162	61	0.0037	Seq. ID No.27
X52001	H.sapiens endothelin 3 mRNA.	89	33	0.0042	Seq. ID No.28
T50788	Unknown	364	102	0.0059	Seq. ID No.38
AJ005866	Putative Sqv-7 like protein	381	163	0.0049	Seq. ID No.29
AF113535	MAID protein	218	100	0.0053	Seq. ID No.39
AB037789	Human cDNA KIAA1368	164	62	0.0092	Seq. ID No.3t
M33987	Carbonic anhydrase	652	46	0.0074	Seq. ID No.31
M77830	Desmoplakin 1 (DPI)	184	61	0.0092	Seq. ID No.32
H81220	EST domain tanscription factor ELF1	113	55	0.017	Seq. ID No.33
AF000592	Human chromosome 21q11-q21 genomic clone	33	69	1.16E-05	Seq. ID No.35
AK021701	Human cDNA FLJ11639	31	63	0.00070	Seq. ID No.36

Example 4: Optimized Portfolio for Colorectal Tumors.

[0050]   The mean-variance optimization algorithm was used to generate a multiple genebased signature, where the genes that are included can be used in combination to distinguish between the normal and tumor samples. Intensity measurements were processed using the samples and microarrays described in Examples 1-3. The data to be analyzed was first preselected based on a pre-specified 5-fold differential between tumor and normal cells. The expression data from genes preselected according to this criteria were then used as follows. The mean and standard deviation of the intensity measurements for each gene were calculated using the non-metastatic samples as the baseline. A discriminating value of X^*(Standard Deviation + Mean) was then calculated for each baseline gene (X was assigned a value of 3). This value was used to ensure the resulting portfolio would be stringent. A ratio of the discriminating value to the baseline value was then calculated for each metastatic sample. This ratio was then converted to a common logarithm. This data was then imported into Wagner Software which produced an efficient frontier from which a portfolio of 4 genes was selected. The set included an unknown sequence, procollagen type I, large subunit of ribosomal protein L21 and fibronectin. These genes are identified as Seq. ID No 42, Seq. ID No. 43, Seq. ID No. 44 and Seq. ID No. 45. Alternatively, a combination of genes used to make up the portfolio can be used to produce diagnostic information that is useful for making clinical decisions regarding colorectal cancer. This is particularly beneficial in the case when a combination of genes selected from the portfolio are combined with additional markers (genetic or not).

Optimized Gene Portfolio:

[0051]

>gil1264443lgblN92134.1lN92134 za23f09.r1 Soares fetal liver spleen 1NFLS Homo sapiens cDNA clone IMAGE:
293417 5' similar to gblM87908lHUMALNE32 Human carcinoma cell-derived Alu RNA transcript, (rRNA); gb:
X57025_mal INSULIN-LIKE GROWTH FACTOR IA PRECURSOR (HUMAN)
>gil2221047lgblAA490172.1lAA490172 ab06b08.s1 Stratagene fetal retina 937202 Homo sapiens cDNA clone
IMAGE:839991 3' similar to gb:J03464 PROCOLLAGEN ALPHA 2(I) CHAIN PRECURSOR (HUMAN)
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>gil834491lgblR62612.1lR62612 yi12d01.s1 Soares placenta Nb2HP Homo sapiens cDNA clone IMAGE:139009
3' similar to gb:X02761_cds1 FIBRONECTIN PRECURSOR (HUMAN);

[0052]   Using a different set of criteria but the same method, a further four gene portfolio was selected by the software.
These are Seq. ID no. 46, Seq. ID No. 47, Seq. ID No. 48 and Seq. ID No. 49. Two genes overlap with the first four-
gene portfolio. The two optimized portfolios can also be combined to form a six-gene portfolio.

Optimized Gene Portfolio:

[0053]

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293417 5' similar to gblM87908lHUMALNE32 Human carcinoma cell-derived Alu RNA transcript, (rRNA); gb:
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12

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tcagctcnct g
431
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Claims

1. A method of assessing colorectal cancer status comprising identifying differential modulation of each gene (relative to the expression of the same genes in a normal population) in a combination of genes selected from the group consisting of Seq. ID. No. 42-49.

2. The method of claim 1 wherein there is at least a 2 fold difference in the expression of the modulated genes.

3. The method of claim 1 wherein the p-value indicating differential modulation is less than .05.

4. The method of claim 1 further comprising employing a colorectal diagnostic that is not genetically based.

5. The method of claim wherein the cancer marker that is not genetically based is selected from the group consisting of carcinomebryonic antigen, CA19-9, CA 125, CK-BB, and Guanylyl Cyclase C.

6. A diagnostic portfolio comprising isolated nucleic acid sequences, their complements, or portions thereof of a combination of genes selected from the group consisting of Seq. ID. No. 42-49.

7. The diagnostic portfolio of claim 6 in a matrix suitable for identifying the differential expression of the genes contained therein.

8. The diagnostic portfolio of claim 7 wherein said matrix is employed in a microarray.

9. The diagnostic portfolio of claim 8 wherein said microarray is a cDNA microarray.

10. The diagnostic portfolio of claim 8 wherein said microarray is an oligonucleotide microarray.

11. A diagnostic portfolio comprising isolated nucleic acid sequences, their complements, or portions thereof of a combination of genes selected from the group consisting of Seq. ID. No. 42-49.

12. A kit for diagnosing colorectal cancer comprising isolated nucleic acid sequences, their compliments, or portions thereof of a combination of genes selected from the group consisting of Seq. ID. No. 42-49.

13. The kit of claim 12 further comprising reagents for conducting a microarray analysis.

14. The kit of claim 12 further comprising a medium through which said nucleic acid sequences, their complements, or portions thereof are assayed.

15. A method of assessing response to treatment for colorectal cancer comprising identifying differential modulation of each gene (relative to the expression of the same genes in a normal population) in a combination of genes selected from the group consisting of Seq. ID. No. 42-49.

16. The method of claim 15 wherein the assessment of the response to therapy includes a determination of whether the patient is improving, not improving, relapsing, likely to improve, or likely to relapse.

17. Articles for assessing colorectal cancer statuts comprising isolated nucleic acid sequences, their complements, or portions thereof of a combination of genes selected from the group consisting of Seq. ID. No. 42-49.

18. Articles for assessing colorectal cancer status comprising representations of isolated nucleic acid sequences, their complements, or portions thereof of a combination of genes selected from the group consisting of Seq. ID. No. 42-49.